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(57) Abstract

Stable lung surfactant compositions are provided, as well as methods for their preparation, modification, formulation, assay, and therapeutic use.

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SURFACTANT COMPOSITIONS AND METHODS

Background of the Invention

This invention relates to lung surfactants, stable surfactant protein compositions and formulations, and methods for the preparation and assay thereof.

Respiratory distress syndrome (RDS), also known as hyaline membrane disease, is a major cause of morbidity and mortality of the prematurely born infant. RDS is believed to be caused primarily by a deficiency of lung surfactant -- a lipid-protein mixture which coats the airspaces of the lung -- thereby reducing the surface tension and preventing airspace collapse. The principal component of lung surfactant -- dipalmitoyl-phosphatidylcholine (DPPC) -- was identified several years ago (Klaus, *et al.*, Proc. Natl. Acad. Sci. USA 47:1858, 1961; Avery, *et al.*, Am. J. Dis. Child. 97:517, 1959). It is believed that administration of lung surfactant to an individual having or at risk of developing RDS is a desirable therapy, and the literature discloses various clinical studies of therapeutic administration of different lung surfactant preparations.

The literature contains various lung surfactant protein preparations, including those with DPPC. Generally, preparations can be classified into five types. These include 1) natural human surfactant (purified from human amniotic fluid), (Merritt, *et al.*, N. Engl. J. Med. 315:787, 1986,), 2) semisynthetic surfactant (prepared by combining DPPC and high density lipoprotein), (Halliday, *et al.*, Lancet 1:476, 1984), 3) animal lung surfactant (isolated by organic extraction of the whole lung or lavage fluid), (Fujiwara, *supra*; Enhorning, *et al.*, Pediatrics 76:145, 1985; Kwong, *et al.*, Pediatrics 76:585, 1985), and 4) purified human surfactant apoproteins (SP-A, SP-B, and/or SP-C purified from natural sources or derived by recombinant DNA technology; see Jobe *et al.*, Am. Rev. Resp. Dis. 136:1032, 1987, and Glasser *et al.*, J. Biol. Chem. 263:10326, 1988) which are reconstituted with surfactant lipids (Revak, *et al.*, J. Clin. Invest. 81:826, 1987).

It has been observed that lung surfactant protein tends to aggregate over time. It is therefore an object of this invention to provide more stable lung surfactant protein compositions and formulations, and methods for making same. It is a further object of this invention to provide lung surfactant protein compositions and formulations with improved biological properties and therapeutic efficacy.

It is also believed desirable for certain therapeutic uses that the surfactant administered have substantially the same structure as natural mammalian surfactant. It is therefore an additional object of this invention to provide lung surfactant protein similar in structure to naturally occurring surfactants.

Summary of the Invention

In the course of their work with lung surfactant, the inventors have discovered that

at least some of the cysteine residues present in lung surfactant protein are esterified to fatty acids in the natural composition, and that this results in reduced aggregation and a more stable formulation.

Accordingly, in one embodiment, methods are provided comprising the modification of the cysteine residues of mammalian (natural or recombinant) lung surfactant protein. Preferred modifications reduce the tendency to aggregation observed of surfactant. These modifications are preferably but not necessarily covalent, and proceed by chemical or enzymatic methods.

Preferred embodiments involve the modification of surfactant protein cysteine residues having free sulfhydryl groups. Surfactant protein is modified with fatty acids or other chemical groups. This modification may proceed so that the cysteine residue is incapable of forming disulfide bonds. In some embodiments, activated derivatives of fatty acids capable of reacting with sulfhydryl groups are reacted with lung surfactant protein having free sulfhydryl groups at the cysteine residues. The resultant reaction product comprises lung surfactant protein containing fatty acid ester groups at its cysteine residues.

In one embodiment, an activated derivative of palmitic acid is used to esterify lung surfactant protein having free sulfhydryl groups at its cysteine residues by reacting the lung surfactant protein with a 4 to 500-molar excess of palmitoyl chloride, preferably a 100 to 400-molar excess of palmitoyl chloride. The reaction is preferably carried out in aprotic solvent to minimize side reactions. The solvents may preferably be mixtures of tetrahydrofuran (THF) and dimethylformamide (DMF) containing one or more acids to lower the pH. A preferred solvent mixture comprised a 50:50 v/v mixture of THF and DMF with 0.1% acid, preferably trifluoroacetic acid. Other acids, such as acetic, phosphoric, or hydrochloric acid may also be employed in the reaction mixture.

In another embodiment, surfactant protein is modified through incubation with a crude extract of lung tissue, or extracts of lung cells such as Type II lung cells or Clara cells under suitable conditions and for a sufficient length of time to palmitoylate cysteine residues in the surfactant protein.

In other embodiments, surfactant protein is analyzed to determine if cysteine residues have been modified. To this end, methods are provided for the mass spectrographic analysis of surfactant protein. In another embodiment, reverse-phase HPLC is provided, utilizing a preferred solvent system of water, butanol, and trifluoroacetic acid (TFA). In yet another embodiment, surfactant protein is analyzed by thin layer chromatography, utilizing a preferred solvent system of butanol, glacial acetic acid, and water. Surfactant protein may also be analyzed by nuclear magnetic resonance.

Still other objects are accomplished through the provision of stable surfactant formulations. Preferred embodiments of such formulations include formulations comprising surfactant protein and reducing agents such as N-acetyl cysteine. In other embodiments,

additional reagents (e.g. other reducing agents or antioxidants) which inhibit disulfide bond formation are included in the formulation.

Surfactant protein modified according to the teachings of this invention may be useful in the prevention and treatment of respiratory distress syndrome. In one embodiment, pharmaceutical preparations are provided which are suitable for administration to mammalian adults or infants having or at risk of developing respiratory distress syndrome, as well as to individuals at risk before their first breath.

Brief Description of the Drawing

FIGURE 1 shows the analysis of HPLC analysis of palmitoylated recombinant SP-C.

Detailed Description of the Invention

Surfactant protein as defined herein means any protein which, when mixed with appropriate lipids, is capable of lowering the surface tension at air-liquid interfaces in the lung. This definition encompasses lung surfactant protein, as described above, together with its amino acid, glycosylation and other variants or derivatives. The literature discussed *supra* describes suitable lung surfactant proteins. It is expected that other surfactant protein variants and derivatives will become available in the future, and these are to be considered to fall within the scope of this invention.

Lung surfactant may be prepared by known methods from synthetic dipalmitoyl-phosphatidylcholine (DPPC), egg or synthetic phosphatidylglycerol (PG), and purified surfactant apoproteins (SP-B and/or SP-C and/or SP-A). Purified surfactant apoproteins are obtained by recombinant methods or direct synthesis using published nucleotide and amino acid sequences (Glasser, *et al.*, Proc. Natl. Acad. Sci. U.S.A. 84:4007, 1987; Jacobs, *et al.*, J. Biol. Chem. 262:9808, 1987; Floros, *et al.*, J. Biol. Chem. 261:9029, 1986; White, *et al.*, Nature 317:361, 1985. Whitsett, *et al.*, Pediatr. Res. 19:501, 1985; Warr, *et al.*, Proc. Natl. Acad. Sci. U.S.A. 84:7[illegible]5, 1987; Hawgood, *et al.*, Proc. Natl. Acad. Sci. U.S.A. 84:66, 1987; Glasser, *et al.*, J. Biol. Chem. 263:9, 1988, Glasser, *et al.*, *supra*, J. Biol. Chem. 263:10326, 1988; and Jobe *et al.*, Am. Rev. Resp. Dis. 136:1032, 1987). Desirably, surfactant apoproteins are reconstituted with surfactant lipids, Revak, *supra*.

Purified surfactant proteins may also be obtained from amniotic fluid, human or animal, or from cell culture, using cells which naturally produce these molecules, such as Type II lung cells or Clara cells. Surfactant protein is also obtained by isolation of natural surfactant from human or animal amniotic fluid. Alternatively, surfactant protein may be isolated by known methods--e.g. by organic extraction of lung tissue or by lavage from human or animal lung, and then supplemented with phospholipids, as desired. Surfactant from other animal species can be used in the treatment of human respiratory distress syndrome, and vice versa.

Fatty acids as used herein refers to non-toxic, straight chain or branched chain compounds, ranging from three to twenty-four carbons; they may be saturated or

unsaturated. Suitable fatty acids include, for example, palmitic acid, oleic acid, linoleic acid, and stearic acid.

Several strategies for the modification of surfactant protein are encompassed by this invention, including covalent modification of cysteine residues with fatty acids or other chemical groups.

In some embodiments, mammalian lung surfactant protein cysteine residues are covalently modified with palmitic acid, resulting in a molecule with a structure similar to that of the native surfactant. Such modifications may be chemical, enzymatic, or the result of direct expression of a modified surfactant protein.

For example, surfactant apoprotein may be chemically modified by reaction with palmityl chloride, as described above and in Example 1. Alternatively, surfactant protein may be modified by reaction with palmitoyl CoA (described for application to myelin proteolipid by Bizzozero *et al.*, J. Biol. Chem. 262:13550, 1987). It may also be modified with activated fatty acid esters; particularly preferred are optionally substituted phenyl-esters of palmitic acid such as nitrophenyl esters, where there are no more than five optional substitutions, which may be nitro, methoxy, halogen, or carboxylate. Preferred derivatives also include palmitate esters of 2-hydroxy pyridine, or 4-hydroxy pyridine. Preparation of these activated derivatives are within the ordinary skill of the art, as shown in C.A. Buehler *et al.*, Survey of Organic Syntheses, page 807, Wiley Book Vol.1 (1970). For example, the reagents above are formed by the reaction of palmitoyl chloride with the appropriate hydroxy-benzene or hydroxy-pyridine derivative. Derivatives of other fatty acids may desirably be made by known methods without undue experimentation. See, e.g., Muzbeck *et al.*, J. Biol. Chem. 264:9716, 1989, and Schultz *et al.*, Ann. Rev. Cell Biol. 4:611, 1988. The reaction of surfactant protein with activated fatty acids is performed in organic solvents in which both reactants are soluble, at approximately 4° - 100°C, for 1-48 hours. Preferably, aprotic organic solvents such as DMF or THF are utilized.

Enzymatic modification of surfactant protein may be accomplished by application of methods known in art. For example, palmitylation or other modifications of surfactant protein may be accomplished by reaction with microsomal membranes from mouse myeloma cell line MPC 11 (Mack *et al.*, J. Biol. Chem. 262:4297, 1987), by reaction with crude cell extracts, membrane extracts or purified protein from other mammalian cells.

In other embodiments, surfactant protein is modified with fatty acids or other chemical groups by a variety of known methods, including but not limited to the following methods: (1) covalent modification using dehydrating or activating agents such as N,N'-dicyclohexylcarbodiimide (DCC) or ethyoxy-ethoxycarbonyl-dihydroquinoline (EEDQ); (2) acylation, using ketenes, anhydrides, isothiocyanates, or beta-lactones; (3) carbamoylation using cyanates; (4) hemimercaptal or hemimercaptol formation using aldehydes and some keto acids; (5) alkylation and arylation, by addition to activated double bonds (using N-

ethylmaleimide), by reactions with quinones, by reaction with haloacids and their amides (using iodoacetic acid, or alpha-bromo-hexadecanoic acid), by methylation reactions (such as with dimethylsulfate), by sulfoalkylation, by arylation (with nitrobenzene compounds), or by reaction with diazo compounds; (6) reaction with metal ions and organic mercury compounds such as mercuric chloride; (7) reaction with arsenic compounds; (8) reaction with sulfites; (9) oxidation reactions; and (10) reaction with sulfenyl halides to form mixed disulfides.

Surfactant protein may be modified within a host cell, or expressed using modified amino-acyl tRNA for site-specific incorporation (see e.g. Noren *et al.*, Science 244:182 (1989)).

This invention also encompasses post-translational modifications to a recombinantly produced protein. It is currently preferred that any post-translational modifications take place within 24, and preferably within a few hours of recovery from a host cell or microorganism. Modifications may also be made to surfactant protein after it is formulated, as will be discussed below. Pharmaceutical compositions comprising surfactant protein modified according to the practice of this invention are encompassed herein, and described in more detail below.

Methods for detecting the presence of modifications to lung surfactant protein are encompassed within the scope of this invention. In one embodiment, the mass spectrum of a sample is obtained and analyzed. Such an embodiment is shown in the examples below.

In other embodiments, thin layer chromatography (TLC) is performed on a sample. While this approach to peptide analysis is common in the field, see e.g. Stuart and Young, Solid Phase Peptide Synthesis, pp 103-107, and 118-122, (Pierce Chem. Co., 2d.ed., 1984), the inventors have discovered an improved method, using a novel solvent system. In a particularly preferred embodiment, 1" x 3" Whatman silica gel plates are used. A 20µl isopropanol extract of cells (~0.3 µg/µl) is loaded onto the TLC plates. A preferred solvent system comprising butanol, glacial acetic acid (HOAc), and H_2O is utilized; while relative proportions of this compounds are envisioned it is presently preferred to utilize volume ratios of 4:1:2. One may use a ninhydrin spray for identification of samples, however different commonly used sprays may be desirable to identify samples containing fatty acids.

In another embodiment, surfactant protein is analyzed by reverse phase HPLC; see e.g. Tomich *et al.*, Analytical Biochemistry 174:197-203 (1988), Rassi, BioChromatography 3:188, 1988, and Johansson *et al.*, FEBS Letters vol 232, no. 1, pp 61-64 (1988). The inventors have discovered novel solvent systems employing an acid, isopropanol, and water, and for certain conditions optionally employing butanol. In one presently preferred embodiment, a Nucleosil C18 column is used, flow rate of 0.5 ml/min, at a preferred temperature of 25°C. Two mobile phases are used: "A" phase comprised of 1 ml trifluoroacetic acid (TFA) and 1000 ml water, and "B" phase comprised of 1 ml TFA, 950 ml isopropanol, and 50 ml

water. A preferred gradient is 58 - 69% "B" in 22 minutes. Absorbance is measured at 214nm. In one embodiment, surfactant analyzed by the foregoing method appeared as a peak at approximately 19 minutes. Use of different acids in the place of TFA is envisioned, such as phosphoric or hydrochloric acid, and different volumes and proportions may be used within the scope of this invention.

In another embodiment of the above reverse-phase HPLC process, a Vydac C4 (#7) column is used, flow rate of 0.5 ml/min, at a temperature of approximately 25°C. As above, two mobile phases are used: "A" phase comprised of 1 ml trifluoroacetic acid (TFA), 300 ml isopropanol, and 800ml water, and "B" phase comprised of 1 ml TFA, 300 ml isopropanol, 200 ml water and 600 ml butanol. For these reagents, a preferred gradient is 0 - 50% "B" in 120 minutes. Absorbance is measured at 228nm, with surfactant analyzed by the foregoing method appeared as a peak at approximately 64.7 minutes. This particular embodiment may be preferred for the analysis of monomeric surfactant.

Different methods of analysis are envisioned with the practice of this invention. An additional example of an HPLC system is described in the Examples below. Further, surfactant protein may be analyzed by NMR.

Surfactant for therapeutic administration is placed into sterile, isotonic formulations together with any desired cofactors. The formulation of surfactant protein is preferably liquid. While conventional parenteral solutions or buffers are usable, it is presently preferred to formulate surfactant protein with excipients which inhibit disulfide bond formation. In one preferred embodiment, surfactant protein is formulated with DPPC, a negatively charged phospholipid containing an unsaturated fatty acid, and a reducing agent or anti-oxidant. Palmitic acid is also desirable. A presently preferred reducing agent is N-acetyl-L-cysteine, however dithiothreitol (DTT), or any of the following commonly used agents may desirably be utilized: inhibitors of disulfide formation (EDTA, or a vial sealed without oxygen and with a nitrogen headspace), tributylphosphine or other tertiary phosphines, mercaptoethanol, D-cysteine, L-cysteine, glutathione (reduced), lipoic acid, coenzyme A, thioredoxin, acyl-carrier proteins that contain 4'-phosphopantetheine, thioglycolic acid, ethylenediamine, maleic acid, sodium metabisulfite or sodium thiosulfate, monothioglycerol, or antioxidants such as catechols, hydroquinones, BHT, ascorbic acid, vitamin E, and gallates. The final concentration of surfactant protein in solution is typically about 1.0-30 mg phopholipid/ml.

In a preferred embodiment, surfactant is formulated according to the following method. Surfactant apoprotein and a mixture of lipids is dried down from organic solvents (evaporation under nitrogen). Typically, the mixture of lipids includes DPPC, 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG), and palmitic acid (PA). The ratio of DPPC to POPG is preferably from 50:50 to 90:10. The ratio of (DPPC + POPG) to surfactant protein is preferably from 50:1 to 5:1. The ratio of PA to (DPPC + POPG) is preferably from 0 to 0.2.

Other unsaturated acidic lipids than POPG are suitable. In a particularly preferred embodiment, the ratio of DPPC to POPG to palmitic acid is 7:3:1, and with a final phospholipid concentration of 3 times the amount of lipid. It is further presently preferred to incorporation calcium into the formulation, at approximately 0.01 mg/ml - 10 mg/ml Ca^{++} (see Chung *et al.*, Biochimica et Biophysica Acta 1002:348-358 (1989)).

After the addition of a first hydration buffer (such as 20mM Na Acetate, pH 6.5, or more preferably 20 mM succinate, pH 6.5, which may contain a reducing agent such as DTT or N-acetyl cysteine in a molar ratio to the surfactant protein of from 2:1 to 600:1 or an antioxidant), the mixture is heated at preferably 65°C for approximately 15 minutes. This dispersion is lyophilized for approximately 12-24 hours. The lyophilized product may be stored at -20°C. The lyophilized dispersion is rehydrated at approximately 37°C for about 15 minutes, in a second hydration buffer which may be water, in a volume equal to that used for the first hydration buffer. The final concentration of surfactant may be from 1.0 - 30 mg phospholipid/ml and is preferably at approximately 10 mg/ml. For embodiments using N-acetyl cysteine as the reducing agent, molar ratios of 100-300:1 (with respect to SP-C) are presently preferred, with 150-250:1 particularly preferred. For embodiments using DTT as the reducing agent, ratios of 100:1 are presently preferred.

Surfactant protein may be provided as lyophilized powder for ultimate delivery in solution. Surfactant protein can also be administered from sustained release compositions, for example as polylactide or polyhydroxylbutyrate implants or liposomes such as are described in EP 17,2007A, or by continuous infusion.

Surfactant protein also is suitably formulated with other commonly known pharmacologic agents in order to modify or enhance the half-life, the distribution, or the therapeutic activity of the surfactant. The surfactant protein formulations may contain agents such as unsaturated or saturated fatty acids, and triglycerides previously suggested for use in surfactant dosage forms (Tanaka, *et al.*, Chem. Pharm. Bull. 31:4100, 1983).

Surfactant protein optionally is administered together with other agents or therapies heretofore employed in the therapy of respiratory distress syndrome. Therapies or agents which are used optionally in a course of therapy with surfactant include, for example, an interferon (including gamma interferon), corticosteroids, thyroid hormone, tocolytics, relaxin, male and female sex hormones, prolactin, insulin, insulin-like growth factor-1, and growth and/or differentiation factors which could induce differentiation of type II cells in fetal lungs and/or increase their surfactant production, such as epithelial growth factor, transforming growth factor beta, or colony stimulating factors. See, for example, Whitsett, *et al.*, J. Biol. Chem. 262:7908 (1987). Other agents include vitamin E, superoxide dismutase, alpha-1-antitrypsin and other antiproteases, selenium, vitamin A, antibiotics, immunoglobulins, and antiviral agents. These other agents or therapies are used at the same time as surfactant is administered or in a sequential course of therapy.

The therapeutically effective dosage of surfactant protein to be employed generally will range about from about 5-900 mg per administration, although the dose will be dependent upon the properties of the surfactant protein employed, e.g. its activity and biological half-life, the concentration of the surfactant protein in the formulation, the rate of dosage, the clinical tolerance of the patients involved, the pathological condition of the patients and the like, as is well within the skill of the physician. It will be appreciated that the practitioner will adjust the therapeutic dose in line with clinical experience for any given surfactant protein.

Surfactant protein may be delivered to the lungs of the fetus transamniotically, and/or to an infant after birth by conventional direct installation after placement of an endotracheal tube. Surfactant protein may be delivered after birth by aerosol, using alternatively a dry power aerosol, a liquid aerosol generated by ultrasonic or jet nebulization, or a metered dose inhaler, again avoiding the complications of endotracheal tube placement.

It should be understood that the Examples below are for illustrative purposes only, and are not to be construed as limiting this invention in any manner.

EXAMPLES

Example 1. Production of Acylated (Palmitoylated) SP-C Using Palmitoyl Chloride.

Human recombinant SP-C (rSP-C) was produced by expression of an SP-C:chloramphenicol acetyltransferase (CAT) fusion protein in *E. coli* and purified by known techniques. The expressed protein was packaged intracellularly as insoluble refractile bodies (inclusion bodies). These bodies were separated from soluble proteins by centrifugation after cell disruption. The inclusion bodies were solubilized in guanidine hydrochloride and the fusion protein was cleaved by addition of hydroxylamine. The concentration of guanidine was reduced by dilution allowing precipitation of the "mature" SP-C. The SP-C was extracted with organic solvents (isopropanol, chloroform:methanol, etc.). Final purification was effected by combinations of gel permeation and reverse phase chromatography.

The rSP-C was solubilized in 50/50 volume/volume dimethyl formamide/ tetrahydrofuran containing 0.1% trifluoroacetic acid (other solvents such as 1-methyl-2-pyrrolidinone are also useful for the reaction). Palmitoyl chloride was then added at 400-fold molar excess compared to SP-C.

The SP-C:palmitoyl chloride mixture was flushed with nitrogen and heated to 37°C for 16 hours. At the end of the reaction, the acylated SP-C was separated by reverse phase HPLC according to the following procedures.

Sample Prep: A 25µl aliquot of the incubation mixture containing 25µg SP-C was dried in the speed vac. The dried sample was taken up in 53µl of 95% isopropanol in 50mM HCl and then diluted with 47µl of 50mM HCl. The final sample was in 100µl of 50% isopropanol in 50mM HCl for injection into HPLC. All samples were incubated overnight at 37°C.

HPLC Column: Reverse phase C8 cartridge (2.1mmx3cm)

Gradient: Buffer A: 5mM HCl

Buffer B: 100% isopropanol in 5mM HCl

35% - 95% Buffer B, 40 min linear gradient

Flow rate: 0.5ml/min

Detection: UV absorbance at 228nM

The HPLC results for the following samples are shown in Fig.1:

1. Control (rSP-C in DMF/THF/TFA)
2. Palmitylated rSP-C (rSP-C + Palmityl chloride in DMF/THF/TFA)
3. Palmityl Chloride Reagent Blank (Palmityl chloride in DMF/THF/TFA)

The recombinant SP-C control eluted at 14 min. After treatment of rSP-C with palmitoyl chloride, several new peaks appeared in the chromatogram. The major new peak eluted at 27.5 min. This elution position corresponds to a dipalmitoylated SP-C (based on elution position of natural bovine SP-C). There is also a minor new OD peak that eluted at 23 min., which may represent monopalmitoylated SP-C. There are several smaller peaks that elute later than 27.5 min. All of the peaks were collected and run on SDS PAGE gels all confirmed to contain SP-C. After treatment with palmitoyl chloride, no SP-C was seen eluting in the position of recombinant SP-C. This indicated that all of the SP-C had reacted with the palmitoyl chloride and was converted to palmitoylated forms. The palmityl chloride reagent blank gave a clean absorbance profile. The peak eluting at 27.5 min. was collected and subjected to mass spectral analysis. The mass obtained was consistent with rSP-C containing two palmitates.

Example 2. Production of Acylated (Palmitoylated) SP-C Using Activated Fatty Acid Esters.

2-(Palmitoyloxy) pyridine hydrochloride was prepared by the reaction of palmitoyl chloride with 2-hydroxypyridine in diethyl ether. rSP-C was obtained as described above and dissolved in DMF/THF (50:50, volume/volume) and a 10- to 400-fold excess of 2-(palmitoyloxy) pyridine hydrochloride. The reaction mixture was incubated at 37°C for 12-48 hours and then analyzed by HPLC. New peaks, more non-polar than SP-C, were collected and were found on mass spectrometric analysis to contain dipalmitoyl derivatives of SP-C.

Example 3. Mass spectrometry analysis of lung surfactant.

Results of mass spectral measurements for SP-C isolated from several species are summarized below. Mass measurements were all made at low resolution, by known methods, and the masses represent the protonated isotopic average mass. Mass accuracy is within 0.8 Da except in cases where the signal intensity is extremely low or resolution of the instrument count not separate overlapping peaks. When mixtures of peptides exist, e.g. N-terminal heterogeneity, all components may not be observed due to ion suppression phenomena.

In this example, palmityl-free SP-C was prepared for mass spectral analysis as follows: dry SP-C, 1-10 μg, was dissolved in 5 μl of 95% isopropanol, 5% trifluoroacetic acid and then vortexed for about 30 seconds. The sample was then concentrated on the mass spectrometer probe and 0.5 μl of m-nitrobenzoic acid was added to the sample probe.

The protocol for removal of palmityl thio esters from SP-C as used in this example was as follows: SP-C, 1-10 μg, was dissolved in 100 μl of freshly prepared cleavage buffer (which consists of 95% isopropanol and 5% 0.2M ammonium bicarbonate and is 0.2M in dithiothreitol). After the addition of cleavage buffer, the reaction tube was flushed with dry nitrogen and then sealed. Cleavage of thioesters was allowed to proceed for 5 hours at 37 °C. Solvent was removed by lyophilization.

1. Canine SP-C

Canine SP-C was obtained and purified as described above. The observed MH+ = 3797.9, and this mass matches the mass expected for the known amino acid sequence (3545.7) plus a palmityl group (presumably a thioester to cysteine) (238.1) plus a methyl ester (14.0) (presumably formed during prolonged storage of the SP-C in chloroform/methanol/HCl.

Treatment of Canine SP-C with DTT for 8 hours at 37°C resulted in an observed MH+ of 3559.0, corresponding to a total release of palmityl.

2. Bovine SP-C

Bovine SP-C was obtained and purified by known methods. The observed MH+ = 4058.7, which matches the mass expected for the amino acid sequence (3582.7) plus 2 palmityl groups (476.2). Treatment of Bovine SP-C with DTT for 8 hour at 37°C resulted in and observed MH+ = 3581.9, corresponding to a total release of palmityl.

3. Human full-term amniotic SP-C

Human full-term amniotic SP-C was obtained and purified by known methods. The observed MH+ matches that expected for the amino acid sequence (3965.2) plus two palmityl groups (476.2).

4. Human alveolar proteinosis SP-C

Human alveolar proteinosis SP-C was isolated and purified from lavage fluid according to known methods. The observed MH+ = 4352.4, which matches that expected for the amino acid sequence (3818.0) plus 2 palmityl groups (476.2) plus isopropyl (42) plus Met-sulfoxide (16). Other peaks in the spectrum: MH+ = 4114.3 (-238.1) represents the equivalent peptide less one palmityl group, MH+ = 3876.2 (-2x238.1) represents the equivalent peptide less two palmityl groups. The isopropyl ester could be formed during prolonged exposure to 95% isopropanol/5mM HCl.

5. Human recombinant SP-C

Human recombinant SP-C was prepared by microbial expression according to known methods. The observed MH+ = 3699.3, which matches that expected for the amino acid sequence (3686.8). Data shows this sample minus any palmityl groups.

Example 4. Imidazole Palmitoylation Methods

This example illustrates an alternative approach which is suitable for acylating the surfactant protein (here SP-C) chemically *in vitro* either before, during or after purification of the protein. The approach described in this disclosure involved acylation of homogenous rSP-C, but the reactions used to acylate the protein could be carried out prior to the last steps of purification, i.e., early in the purification scheme.

The palmitoylation reaction involves transfer of palmitic acid from the reagent N-palmitoylimidazole to the cysteine residues of SP-C. The reaction is catalyzed by excess imidazole. The acyl thioesters of SP-C were then purified by reverse phase HPLC.

The N-palmitoylimidazole was synthesized essentially as described by Cronan and Klages, PNAS 78:5440-5444 (1981). Palmitoyl chloride was added to twice the molar ratio of imidazole dissolved in benzene. The mixture was stirred for at least 3 hours and then filtered to remove the insoluble imidazolium chloride. The benzene was then removed by evaporation under vacuum. The residue was dissolved in ethyl acetate for subsequent recrystallization. The resulting N-palmitoylimidazole was used in subsequent reactions as the fatty acyl donor.

The initial acylation experiments were carried out in aqueous buffers using detergents to solubilize the SP-C. In these experiments, SP-C was dried under vacuum and redissolved in 10% Nonidet P-40 (Sigma). A 9-fold molar excess of palmitoylimidazole was added, and also enough imidazole to bring the concentration to approximately 500 mM. This 9-fold excess is an 18-fold molar excess with respect to the cysteines (2 cys/SP-C). The pH of the reaction was adjusted to 6.5. Dithiothreitol (DTT) (equimolar compared to SP-C) was also added. The reaction was allowed to proceed for fourteen hours with aliquots removed at 30 minutes and one hour for interim analysis.

Before analysis, aliquots from each time point were dried under vacuum and then solubilized in 35% isopropanol containing 5% 0.1 n HCl. These samples were then chromatographed over either Vydac C4 or C8 reverse phase HPLC columns. This technique allows resolution of the various species of palmitoylated SP-C. The conditions for the chromatography were as follows: buffer A -- 5 mM HCl, buffer B -- 100% isopropanol in 5 mM HCl, 40 minute gradient 35%-95% buffer B. The flow rate was 1 ml/min with the detector set at 228 nm.

The HPLC separation allows resolution of 4 separate peaks of acylated SP-C (data not shown). These peaks represent increasing numbers of palmitate groups (up to four) on SP-C. The SP-C containing four palmitate groups elutes last in the gradient (most hydrophobic) (peak 4) while the SP-C derivatized with only one palmitate elutes first (peak one). The results from this experiment can be summarized as follows: Aliquots taken early in the reaction (30 minutes and 1 hour) contained peak 1 and peak 2. These peaks contain SP-C with one and two palmitates respectively. With time (14 hours) peaks one and two decreased

in magnitude while two additional peaks, 3 and 4, appeared. These peaks correspond to SP-C containing 3 and 4 palmitates. Approximately 75% of the palmitates on SP-C which elute in peak 1 and 2 are linked to cysteines. The remaining acylation is presumably to the amino groups on the n-terminal glycine and the epsilon amino group of lysine. These estimates are based on the ability of reducing agents to deacylate palmitates on cysteines but not on amino groups. We also developed methods for cysteine acylation in nonaqueous solvent systems. In one method the SP-C was directly acylated with N-palmitoylimidazole in 95% isopropanol containing 5% 0.1 n HCl. The concentration of the acylating reagent was varied over a range of concentrations from 1 to 20 fold molar excess compared to SP-C (2-40 fold over the cysteine thiols). The time course of the reaction was also varied from 30 minutes to 16 hours. Again shorter reaction times favor the specific acylation of cysteine, while increasing times lead to the formation acyl-amino groups.

CLAIMS

We claim:

1. A method for the modification of lung surfactant protein, comprising:
   a) preparing a crude extract of the lung tissue of a mammal in aqueous suspension; and
   b) reacting said crude extract with lung surfactant for a sufficient length of time to permit the palmitoylation of said lung surfactant.
2. A method for the modification of lung surfactant protein, comprising:
   a) preparing a crude extract in aqueous suspension of mammalian lung cells; and
   b) reacting said crude extract with lung surfactant for a sufficient length of time to permit the palmitoylation of said lung surfactant.
3. The method of claim 2, wherein said lung cells are selected from the group consisting of Type II lung cells and Clara cells.
4. The method of claim 2, wherein said crude extract is prepared from the membranes of said lung cells.
5. The method of claim 2, wherein said crude extract is comprised of a detergent extract of said lung cells.
6. A method for the modification of lung surfactant protein, said lung surfactant protein having a cysteine residue, comprising the modification of said cysteine residue so that said residue is incapable of forming disulfide bonds.
7. The method of claim 6, wherein said cysteine residue bears a free sulfhydryl group.
8. The method of claim 6, wherein said modification is covalent.
9. The method of claim 6, wherein said modification comprises reacting said surfactant protein with an activated derivative of a fatty acid for a sufficient length of time to permit the esterification of said fatty acid with said surfactant.
10. The method of claim 9, wherein said fatty acid has C_3 to C_{24} carbons.
11. The method of claim 9, wherein said modification comprises reacting said surfactant protein with an activated derivative of palmitic acid for a sufficient length of time to permit the palmitoylation of said lung surfactant.
12. The method of claim 11, wherein said derivative is a phenyl ester of palmitic acid.
13. The method of claim 11, wherein said derivative is a pyridine derivative of palmitic acid.
14. A method for the modification of lung surfactant protein having a cysteine residue, comprising the reaction of said surfactant protein with a reagent selected from the group consisting of palmitoyl chloride, palmitoyl CoA, and activated fatty acid esters.
15. The method of claim 14, wherein said lung surfactant protein is synthetic.
16. The method of claim 14, wherein said surfactant protein is produced by recombinant expression.

17. The method of claim 14, comprising the additional step of recovering said surfactant protein under reducing conditions and thereafter modifying said cysteine residue.

18. The method of claim 14, wherein said surfactant protein is recovered and modified within a time course sufficient for inhibiting formation of disulfide bonds.

19. A pharmaceutical composition usable for the treatment of respiratory distress comprising an effective amount of a lung surfactant protein, a reducing agent, and a pharmaceutically effective carrier.

20. The composition of claim 19, further comprising a negatively charged phospholipid having an unsaturated fatty acid.

21. The composition of claim 19, further comprising a saturated fatty acid.

22. The composition of claim 19, wherein said reducing agent is N-acetyl cysteine.

23. The composition of claim 19, further comprising palmitate.

24. The composition of claim 19, further comprising dipalmitoyl phosphatidylcholine.

25. The composition of claim 19, further comprising palmitoyl-oleoyl-phosphatidylglycerol.

26. A method for treating respiratory distress comprising administering to a patient having or at risk of having respiratory distress an effective amount of the composition of claim 19.

27. A method for assaying lung surfactant protein, comprising the steps of:
(a) providing a sample of lung surfactant protein; and
(b) determining the presence of fatty acid esters with the surfactant protein.

28. The method of claim 27, wherein said fatty acid ester is a palmitic acid ester.

29. The method of claim 27, wherein said presence is determined by thin layer chromatography, utilizing a solvent system comprising HOAc, H_2O, and butanol.

30. The method of claim 27, wherein said presence is determined by reverse phase HPLC, utilizing a solvent system comprising an acid, isopropyl alcohol, and H_2O.

31. The method of claim 30, wherein said acid is selected from the group consisting of TFA, HCl, and phosphoric acid.

32. The method of claim 27, wherein said presence is determined by obtaining the mass spectrum of said sample.

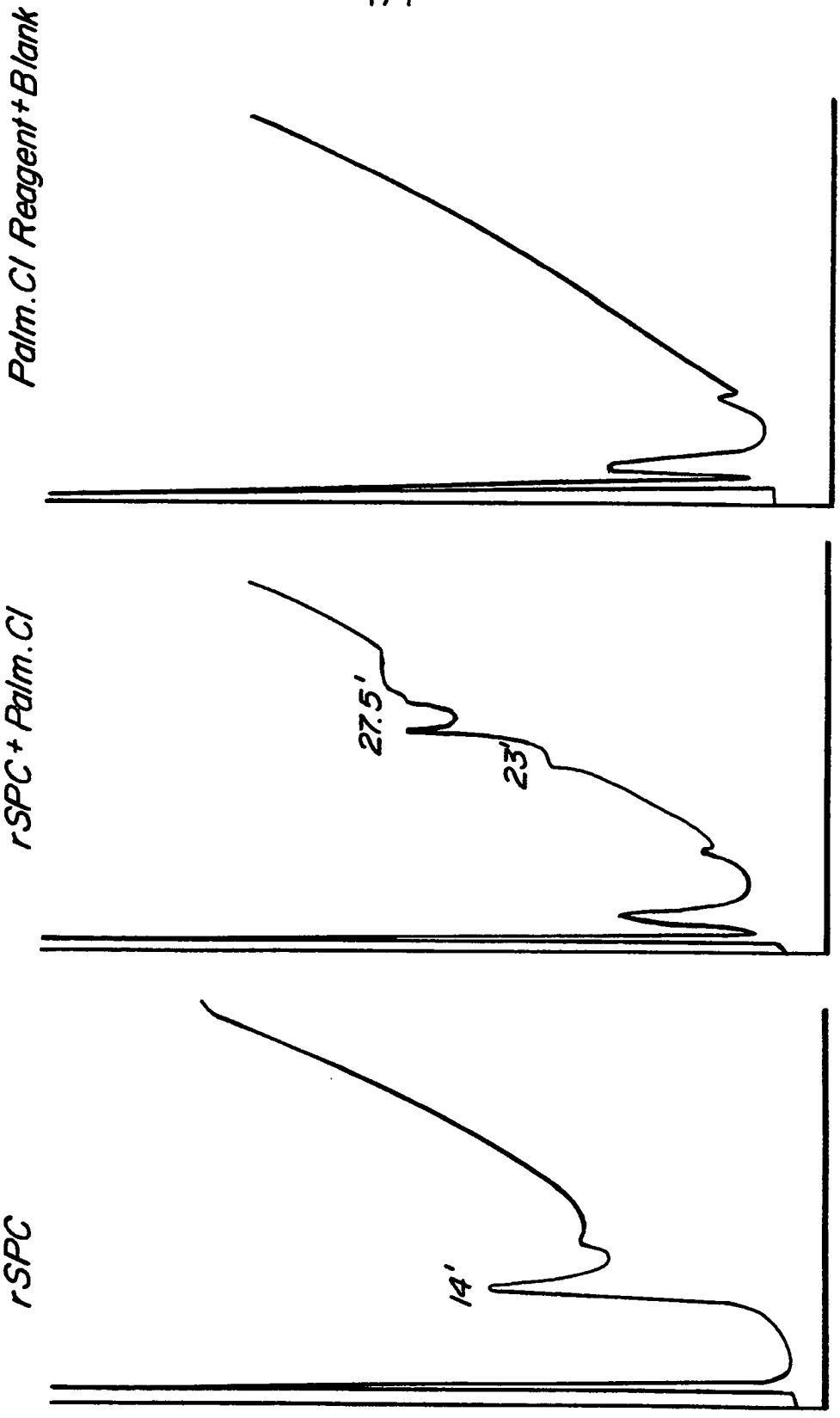

FIG. IC

FIG. IB

FIG. IA

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/03856

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 07 K 13/00, A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 07 K, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Chemical Abstracts, volume 109, no. 21, 21 November 1988, (Columbus, Ohio, US) Y. Kuroki et al.: "Chemical modification of surfactant protein A alters high affinity binding to rat alveolar type II cells and regulation of phospholipid secretion" see page 477, abstract no. 187827j & J. Biol. Chem. 1988, 263(33), 17596-602 --	1
A	Chemical Abstracts, volume 105, no. 8, 25 August 1986, (Columbus, Ohio, US) see page 379, abstract no. 66463w & JP, A, 6165821 (TOKYO TANABE CO., LTD.) 4 April 1986 --	20-25
P,X	EP, A, 0368823 (KABIGEN) 16 May 1990 see the whole document, especially examples 7-8; claims 1-6 ----	1-18

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5th November 1990	29. 11. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Natalie Weinberg

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 26, because they relate to subject matter not required to be searched by this Authority, namely:

see rule 39(IV)PCT: methods for treatment of the human body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers_____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers_____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9003856 SA 39084

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 20/11/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0368823	16-05-90	AU-A- 4299689	26-04-90
		JP-A- 2145599	05-06-90
		SE-A- 8803713	18-10-88